Direct and rapid genotyping of glutathione-S-transferase M1 and T1 from human blood specimens using the SmartAmp2 method.

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Abbreviations;
SmartAmp-2; Smart Amplification Process version 2, GST; Glutathione S-transferase, DILI; Idiosyncratic drug-induced liver injury, γ-GCS; γ-glutamylcysteine synthetase
Abstract

Clinical studies have suggested that a defect in both glutathione S-transferase (GST) M1 and GSTT1 increases the risk of drug-induced hepatotoxicity. The present study developed the method allowing genotyping of GSTM1 and GSTT1 directly using a small aliquot of blood samples based on an isothermal Smart amplification process ver.2 (SmartAmp-2). SmartAmp-2 reaction could complete the genotyping of GSTM1 and GSTT1 within 40 min. The population of wild-type, GSTM1 null, GSTT1 null and double null was 24, 21, 35 and 19%, respectively, and consistent with previous reports in Japanese population. The genotypes determined by SmartAmp-2 using ninety-four human blood specimens perfectly matched those using the corresponding genomic DNA, and the genotypes determined by the conventional PCR method. The SmartAmp-2 method enables rapid identification of the genotypes of GSTM1 and GSTT1, saving time and effort for the genomic DNA preparation and electrophoresis in clinical practice, and will contribute to evaluate the susceptibility of disease and adverse reactions to drugs caused by deletion of GSTM1 and GSTT1.
Introduction

Idiosyncratic drug-induced liver injury (DILI) is a clinical challenge because of the rarity of its diagnosis and the lack of a standard, which makes determination of causality difficult (Kaplowitz, 2005). Many drugs have been withdrawn from the market or clinical development of new drugs has suddenly been halted because of DILIs (Kola and Landis, 2004). Formation of reactive metabolites, electrophilic intermediates, is attributed to DILI (Walgren et al., 2005). Drugs are metabolized by CYP enzymes to reactive metabolites which form covalent bonds with macromolecules. Then, the drug-protein adducts can inhibit the functions of vital intracellular proteins or stimulate the immune system, leading to DILI.

The liver has protective mechanisms against such reactive metabolites. They are detoxified by conjugation with glutathione (GSH) catalyzed by Glutathione S-transferases (GSTs). GSTs comprise a supergene family and catalyze the detoxification of a variety of reactive compounds, chemicals and their metabolites (Eaton and Bammler, 1999; Hayes et al., 2005). The importance of this glutathione detoxification system has been proposed in γ-glutamylcysteine synthetase-knockdown rats (Morita et al., 2009) and Nrf2-knockout mice (Enomoto et al., 2001) which were sensitive to the DILI produced by diclofenac, flutamide and acetaminophen.

There is growing interest in GSTM1 and GSTT1 genotyping as risk factors for
DILI. GSTM1 and GSTT1 are predominantly expressed in the liver and display polymorphisms in humans. In the double-null variant of GSTM1 and GSTT1, 16 kb or 52 kb are deleted from chromosome 1 or chromosome 22 by a homologous recombination, respectively (Figure 1) (Xu et al., 1998; Sprenger et al., 2000). The double null variant was associated with a higher risk of DILIs caused by troglitazone, tacrine and carbamazepine (Simon et al., 2000; Watanabe et al., 2003; Ueda et al., 2007).

A simple and rapid detection of GSTM1 and GSTT1 genotypes before medication will help avoid severe DILI in patients in clinical practice. In the present study, we developed a rapid polymorphism detection system, the Smart Amplification process version 2 (SmartAmp-2®), for GSTM1 and GSTT1 genes. SmartAmp-2 enables genotyping of the genes under isothermal conditions using small aliquots of blood specimens, which could save time and effort by avoiding genomic DNA preparation and electrophoresis (Mitani et al., 2007). In the SmartAmp-2 reaction, the folding primer (FP) and turn-back primer (TP) participate in primer extension events, producing an intermediate single stranded DNA product. These intermediate species then undergo a self-priming reaction to generate long DNA concatemeric products. Boost primer (BP), outer primer 1 (OP1) and outer primer 2 (OP2) support functions of FP and TP and
therefore increase the reaction speed. The non-specific amplification can be totally suppressed by preventing generation of unexpected intermediate products such as primer-dimer, or inhibiting amplification from non-target sequences.

In the present study, we designed the 5 primers for the detection of GSTM1 and GSTT1 genes, and validated their specificity and reliability using a hundred human genomic DNA specimens.
Methods

Collection of genomic DNA and blood, and preparation of a template

Peripheral blood was collected from 100 healthy Japanese volunteers, and DNA was extracted by the standard protocol. The samples were numbered, unlinked, and tested anonymously. This study was approved by the Institutional Ethics Review Boards, and written informed consent was obtained from all subjects before the study. Two volumes of 50mM NaOH were added to one volume of each of the blood specimens. A panel of human genomic DNA was obtained through Coriell Cell Repositories (NJ, USA) consisting of European-American, African-American, Mexican-American, Native American and Asian-American specimens. The DNA concentration is about 10ng/μL.

The SmartAmp-2 Assay for GSTM1 and GSTT1 Polymorphism Detection using human blood specimens and genomic DNA

SmartAmp primer sets were designed for amplification and detection of GSTM1 and GSTT1 genes (Figure 1). After genomic DNA and blood specimens were denatured at 98°C for 3 and 5 min, respectively, SmartAmp-2 reactions were allowed to take place at 60°C for 60min. The Mx3000P™ Real-time PCR system (Agilent technologies, USA) and LightCycler® 480 System II (Roche) were used for maintaining isothermal
conditions and monitoring the change in fluorescence intensity of intercalating SYBR®
Green I dye (Molecular Probes, USA) during the reaction.

The reaction mixture contains 2.9μM each of FP and TP, 1.5μM BP, 0.36μM each of OP1 and OP2, 1.4mM dNTPs, 5% DMSO, 20mM Tris-HCl (pH 8.0), 10mM KCl, 10mM (NH₄)₂SO₄, 8mM MgSO₄, 0.1% Tween® 20, 1/100,000 diluted original SYBR®
Green I, 6 units of Aac DNA polymerase (DNAFORM K.K.) and prepared genomic DNA or blood. For the quality of control of the reaction, the SmartAmp-2 kit for EGFR was used. Primers: TP; 5’CACCGCAGCAGTGGTCCGCACCCAGTGGTG3’, FP; 5’CACCTTCACCCTCAGAAGGTGACCTGCAGCCAGGAACG3’, BP; 5’ACAGATTGGGGCT3’, OP1; 5’GACCGTCGCTTGGTGCAC3’, OP2; 5’CCTCCTTCTGCATGGTAT3’.

Genotyping of GSTM1 and GSTT1 of Genomic Samples by the conventional PCR method.

Genotyping of GSTM1 and GSTT1 was carried out by conventional PCR amplification as described previously (Watanabe et al., 2003; Bernardini et al., 2005).

GSTM1 Primers: sense; 5’GAACCTCCCTGAAAAAGCTAAG 3’, antisense; 5’GTTGGGCTCAAATATACGGTG3’, GSTT1 Primers: sense; 5’
TTCCTTACTGGTCCTCACATCTC 3’, antisense; 5’

TCACCGGATCATGGCCAGCA 3’,

The PCR was carried out for 35 cycles of 30 seconds at 94°C, 30 seconds at 60°C, 30 seconds at 72°C. The amplified DNA fragments were electrophoresed in 2.0% agarose gel, stained with ethidium bromide, and visualized under ultraviolet illumination. The amplified fragments detected at 0.46- or 0.23-kb were designated as wild type GSTM1 or GSTT1 genes, respectively.
Results and Discussion

The primer sets of GSTM1 and GSTT1 were designed to exon 5 and intron 4, respectively, which are not conserved among members of the GST genes. Screening of 5 primer combinations in primer sets yielded an ideal primer set which completed the amplification within 30 min and 40 min for the genomic DNAs and blood specimens, respectively. A representative amplification profile is shown in figure 1. After 40 min, the GSTM1 or GSTT1 genes could be detected without non-specific amplification in the signals produced by self- non-specific amplification until 60 min. No amplification when the genomic DNA specimens of GSTM1 null or GSTT1 null was used as template shows the specificity of the amplified signals.

With this kit, genotyping of GSTM1 and GSTT1 were carried out, using ninety-four blood samples (Table 1). First of all, accuracy was checked using commercially available human genomic DNAs, then validation was performed using peripheral blood specimens and the corresponding genomic DNAs. Conventional PCR was carried out to analyze the genotype of GSTM1 and GSTT1 using the corresponding genomic DNA specimens. The genotypes were matched without false positives or false negatives. The population of wild-type, GSTM1 null, GSTT1 null and double null was 24%, 21%, 35% and 19%, respectively, and consistent with previous reports. Since
genotypes of GSTM1 and GSTT1 were matched by a conventional method and the SmartAmp-2 assay, we can conclude that this new SmartAmp-2 assay is reliable.

Previously, the genotyping of GSTM1 and GSTT1 was carried out by conventional PCR followed by electrophoresis, and real-time PCR. The most striking difference in the SmartAmp-2 assay compared with these conventional methods is that SmartAmp-2 can save time and avoid genomic DNA preparation and electrophoresis because genotyping can be completed within 40 min including sample preparation. Furthermore, since the reaction in the SmartAmp-2 assay occurs under isothermal conditions, we can perform genotyping with only a simple device for warming and a detection system. Furthermore, new primers applicable to SmartAmp-2 were launched, referred to as Exciton Primers capable of visual detection of the end product, and multiplex labeling of the end products in one test tube (Lezhava et al., 2010), a further saving of money, labor and time. These inventions will allow medical staff to determine these genotypes even at the bed-side in the future. It should be noted that, because of a lack of detailed information about the exact region where GSTM1 and GSTT1 are deleted, we were unable to design primers for the detection of a null mutation to distinguish homozygotes from heterozygotes. SmartAmp-2 was unable to discriminate the copynumber variation of GSTM1 (McLellan et al., 1997). However,
since most clinical studies have compared the incidence of DILI in patients classified into the two groups, null and others, the present system satisfies the clinical needs.

Until now, deletion of GSTM1 and GSTT1 has been associated with the susceptibility to DILI caused by troglitazone, tacrine and carbamazepine (Simon et al., 2000; Watanabe et al., 2003; Ueda et al., 2007). In addition, recent clinical studies have indicated that it is also associated with a susceptibility to different forms of bladder cancer, breast cancer, and prostate cancer (OR=1.44, 2.03 and 1.33, respectively) (Engel et al., 2002; Kostrykina et al., 2009; Mo et al., 2009). Therefore, in addition to medication, genotyping of GSTM1 and GSTT1 will become more important in the future to assess the cancer susceptibility of patients. With regard to this, the SmartAmp-2 assay has a major advantage: medical staff can perform genotyping at the bedside using a simple system, or handle large number of samples using 96- and 384 well plates and the current real-time PCR system.

In conclusion, a SmartAmp-2 assay for the genotyping of GSTM1 and GSTT1 was developed in this study, and the reliability of the method was validated using the conventional method. The system will allow evaluation of the susceptibility to disease and adverse reactions to drugs caused by deletion of GSTM1 and GSTT1 in clinical situations.
References


Footnotes

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**Legend for figure**

**Figure 1 Genotyping of GSTM1 and GSTT1 genes by SmartAmp-2.**

The target regions of GSTM1 and GSTT1 genes and primers for SmartAmp-2 assay are illustrated. In the representative amplification-time profiles by SmartAmp-2 assay using human blood specimens, amplification of GSTM1 GSTT1 and EGFR are indicated by closed circles, squares and triangles, respectively. dR, Δ raw fluorescence.
Table

Table 1. Comparison of SmartAmp-2 with the conventional PCR method.

The genotypes of GSTM1 and GSTT1 were analyzed by both the conventional PCR method and the SmartAmp-2 method. The table shows a comparison of the two methods in 101 genomic DNA samples and in 94 blood samples.

<table>
<thead>
<tr>
<th>SmartAmp-2 assay</th>
<th>Genomic DNA samples</th>
<th>Blood samples</th>
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<tbody>
<tr>
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Figure 1

Primers of GSTM1 and GSTT1

Amplification profile of SmartAmp-2

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